Bromodomain inhibitors correct bioenergetic deficiency caused by mitochondrial disease complex I mutations

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Abstract: Mitochondrial diseases comprise a heterogeneous group of genetically inherited disorders that cause failures in energetic and metabolic function. Boosting residual oxidative phosphorylation (OXPHOS) activity can partially correct these failures. Herein, using a high-throughput chemical screen, we identified the bromodomain inhibitor I-BET 525762A as one of the top hits that increases COX5a protein levels in complex I (CI) mutant cybrid cells. In parallel, bromodomain-containing protein 4 (BRD4), a target of I-BET 525762A, was identified using a genome-wide CRISPR screen to search for genes whose loss of function rescues death of CI-impaired cybrids grown under conditions requiring OXPHOS activity for survival. We show that I-BET525762A or loss of BRD4 remodeled the mitochondrial proteome to increase the levels and activity of OXPHOS protein complexes, leading to rescue of the bioenergetic defects and cell death caused by mutations or chemical inhibition of CI. These studies show that BRD4 inhibition may have therapeutic implications for the treatment of mitochondrial diseases.

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Article

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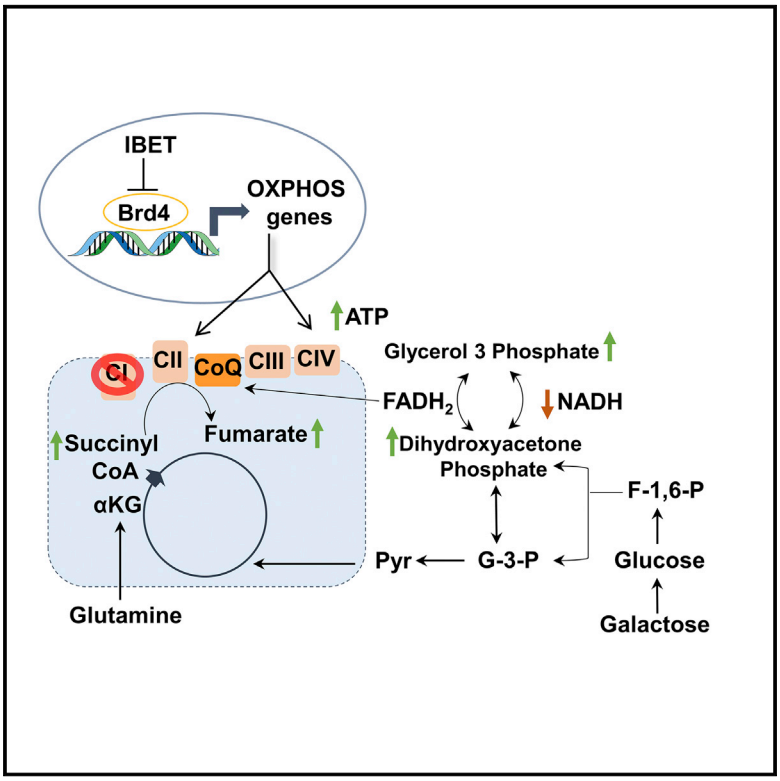
Graphical Abstract



Authors

Joeva J. Barrow, Eduardo Balsa, Francisco Verdeguer, ..., John Doench, David E. Root, Pere Puigserver

Correspondence

pere_puigserver@dfci.harvard.edu

In Brief

Mitochondrial diseases cause failures in energetic function. Boosting oxidative phosphorylation (OXPHOS) activity can be therapeutic. Barrow et al. (2016) demonstrate through high-throughput chemical and CRISPR genetic screens that the protein BRD4 can be a therapeutic target. Inhibiting BRD4 increases OXPHOS capacity, rewires the metabolome, and rescues complex I mutant cells from death.

Highlights

- Bromodomain inhibitors or BRD4 ablation boosts OXPHOS capacity in CI mutant cells
- Inhibition of BRD4 rescues CI mutant cells from galactose-induced cell death
- BRD4 binds to OXPHOS promoters and competes with PGC-1α binding
- Bromodomain inhibition rewires the metabolome in CI mutant cells to preserve ATP

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Bromodomain Inhibitors Correct Bioenergetic Deficiency Caused by Mitochondrial Disease Complex I Mutations

Joeva J. Barrow,[1,2,5] Eduardo Balsa,[1,2,5] Francisco Verdeguer,[1,2] Clint D.J. Tavares,[1,2] Meghan S. Soustek,[1,2] Louis R. Hollingsworth IV,[1] Mark Jedrychowski,[2] Rutger Vogel,[1,2,4] Joao A. Paulo,[2] Jan Smeitink,[4] Steve P. Gygi,[2] John Doench,[3] David E. Root,[3] and Pere Puigserver[1,2,3,6,*]
[1]Department of Cancer Biology, Dana-Farber Cancer Institute
[2]Department of Cell Biology, Harvard Medical School
Boston, MA, USA 02215
[3]Broad Institute of MIT and Harvard, Cambridge, MA, USA 02142
[4]Radboud Center for Mitochondrial Medicine, Radboud University Medical Center, Nijmegen 6500HB, the Netherlands
[5]Co-first author
[6]Lead Contact
*Correspondence: pere_puigserver@dfci.harvard.edu
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SUMMARY

Mitochondrial diseases comprise a heterogeneous group of genetically inherited disorders that cause failures in energetic and metabolic function. Boosting residual oxidative phosphorylation (OXPHOS) activity can partially correct these failures. Herein, using a high-throughput chemical screen, we identified the bromodomain inhibitor I-BET 525762A as one of the top hits that increases COX5a protein levels in complex I (CI) mutant cybrid cells. In parallel, bromodomain-containing protein 4 (BRD4), a target of I-BET 525762A, was identified using a genome-wide CRISPR screen to search for genes whose loss of function rescues death of CI-impaired cybrids grown under conditions requiring OXPHOS activity for survival. We show that I-BET525762A or loss of BRD4 remodeled the mitochondrial proteome to increase the levels and activity of OXPHOS protein complexes, leading to rescue of the bioenergetic defects and cell death caused by mutations or chemical inhibition of CI. These studies show that BRD4 inhibition may have therapeutic implications for the treatment of mitochondrial diseases.

INTRODUCTION

Mutations in the mitochondrial or nuclear DNA that compromises the oxidative phosphorylation (OXPHOS) system lead to a spectrum of debilitating or even fatal human disorders known as mitochondrial diseases (Koopman et al., 2012). Among them, mitochondrial complex I (CI) deficiency is the most common OXPHOS defect observed in patients, and to date no cure is available (Pfeffer et al., 2013; Swalwell et al., 2011). The impairment of OXPHOS due to dysfunction in the electron transport chain (ETC) compromises ATP production (Nunnari and Suomalainen, 2012) and, depending on the mutation or insult, increases the generation of reactive oxygen species (ROS) (Lin et al., 2012; Vafai and Mootha, 2012) and unbalances the NAD^+ to NADH ratio due to NADH accumulation (Karamanlidis et al., 2013). Proposed metabolic strategies to correct mitochondrial CI deficiencies include using mitochondria-targeted antioxidant molecules (Koopman et al., 2016) or biochemically bypassing the defective complex—for example, using succinate (Pfeffer et al., 2013) or short-chain quinones (idebenone or CoQ1) (Haefeli et al., 2011) that can feed electrons into the ETC downstream of CI.

The validity of striving to boost residual mitochondrial activity to overcome bioenergetic defects has recently been strengthened by several studies reporting that overexpressing the transcriptional coactivator PGC-1α (a known central regulator of mitochondrial biogenesis) partially corrects pathological phenotypes and extends survival in mouse models with ETC deficiencies (Dillon et al., 2012; Srivastava et al., 2007; St-Pierre et al., 2006). Based on these findings, a possible approach to overcome ETC deficiencies is to enhance the functional OXPHOS capacity which is the failing hallmark of these diseases.

Bromodomain-containing protein 4 (BRD4) is a member of the bromodomain and extraterminal domain (BET) family of proteins, comprised of BRD2–BRD4 and BRDT (Nicodeme et al., 2010). BET proteins contain two tandem bromodomains (protein modules that bind to acetyl-lysines) and an extraterminal domain (ETD) that mediates protein-protein interactions (Dhalluin et al., 1999). BRD4 binds to acetylated histones and coordinately recruits additional proteins via its ETD to promoters and distal enhancers to modulate gene expression (Liu et al., 2013). Chemical inhibitors to the BET family such as I-BET 525762A and JQ1, which occupy the epsilon acetyl-lysine binding pocket of BRD4 and prevent its association to acetylated histones at the chromatin, have been effective in treating several cancer types (Dawson et al., 2011; Delmore et al., 2011; Filippakopoulos et al., 2010). However, it is unknown whether BRD4 can control genes linked to energy metabolism and impact ETC deficiencies.

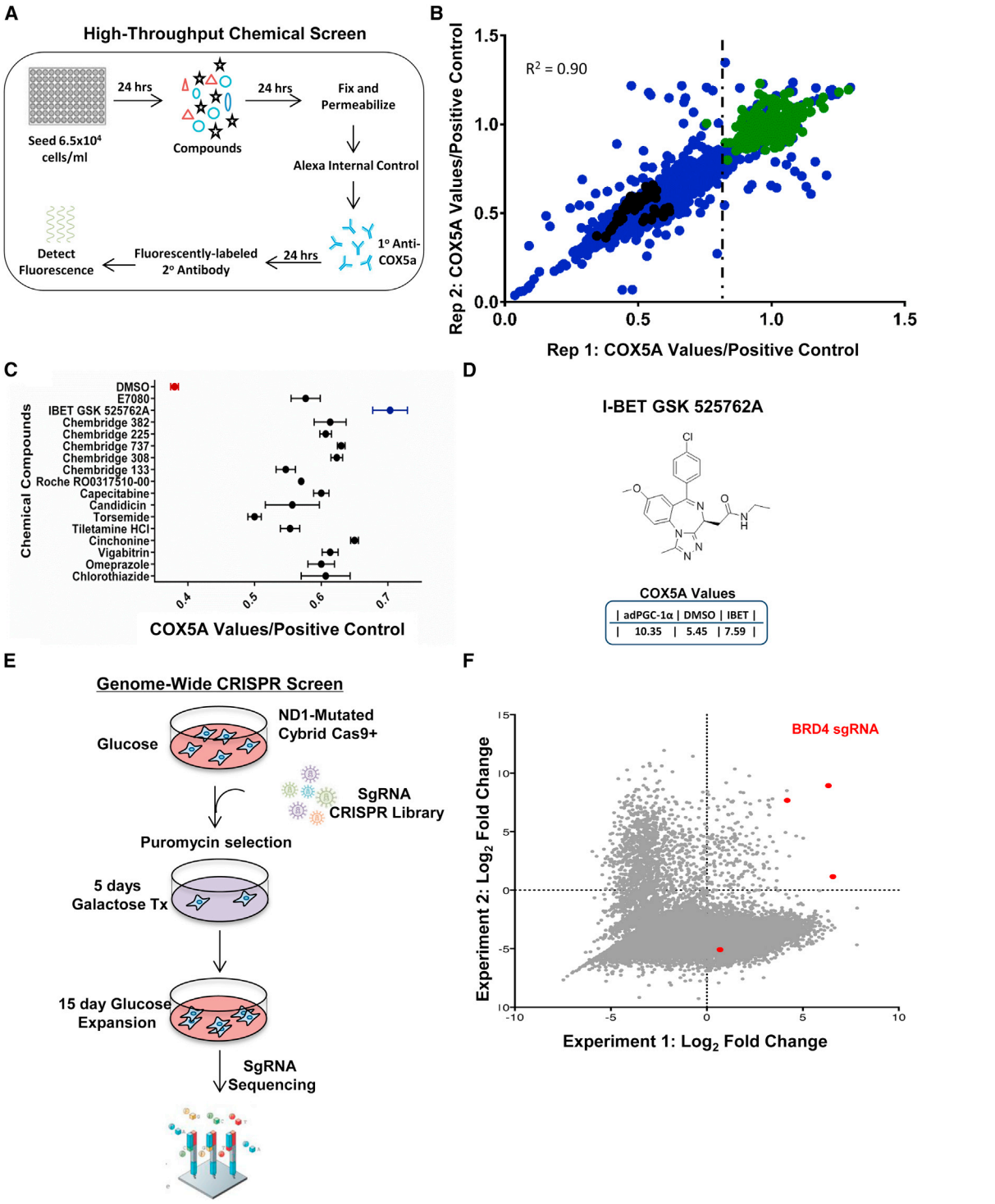

Figure 1. BRD4 Is Identified by Both High-Throughput Chemical and CRISPR Genetic Screens as a Target to Enhance OXPHOS Proteins and Rescue Mitochondrial Bioenergetic Defects

(A) Schematic of high-throughput chemical screen.

(B) Scatterplot of chemicals tested (blue) plotted with first test (x axis) and repeated test (y axis) of COX5a fluorescent values relative to the average PGC-1α-positive control (green). DMSO baseline fluorescent values (black) are indicated. Vertical dotted line indicates compounds selected at the 70% cutoff.

(C) COX5a fluorescent values from selected compounds tested in secondary screen plotted relative to PGC-1α positive control set to 1.0. Values represent the mean ± SEM. n = 3.

(legend continued on next page)

Here we have identified BRD4 using a mitochondrial-based high-throughput chemical screen and tandem-genome wide-CRISPR screen in human CI mutant cybrid cells. BRD4 inhibition, either chemically or genetically, rescues mitochondrial bioenergetics, protecting against cell death caused by CI defects. Deletion or inhibition of BRD4 enhances OXPHOS genes, proteins, and activity, increasing $FADH_2$ levels to bypass defective CI. These studies show that BRD4 inhibition corrects mitochondrial CI deficiencies and may have therapeutic implications for the treatment of mitochondrial diseases.

RESULTS

Identification of a Bromodomain Inhibitor and BRD4 in High-Throughput Chemical and Genome-wide CRISPR Screens

In order to discover chemical compounds that rescue bioenergetic defects caused by mitochondrial disease mutations through increases of mitochondrial proteins, we designed and developed a high-throughput in-cell enzyme-linked immunoassay using human cybrid cells carrying a mutation (3796 A>G, found in adult onset dystonia) in the mitochondrial-encoded protein ND1—an integral component of the NADH dehydrogenase CI subunit (Simon et al., 2003) (Figure 1A). A diverse library of 10,015 chemical compounds were screened in duplicate, and values were normalized to cells expressing PGC-1α—a transcriptional regulator of mitochondrial biogenesis (Puigserver et al., 1998; Wu et al., 1999) as a positive control (Figure 1B). CIV was the most responsive to PGC-1α-stimulation; therefore, the quantitative measurement of the CIV subunit COX5a served as the readout. A 70% threshold was established to select top hits for re-test using the same assay. Interestingly, the compound with the highest score was I-BET 525762A, a pan-BET inhibitor that targets the BET family of proteins including BRD2–BRD4 and BRDT (Nicodeme et al., 2010) (Figures 1C and 1D). In parallel, and to complement this chemical screen, we performed a genome-wide editing CRISPR screen. We used the standard cell-death-based clinical assay to culture ND1 mutant cybrid cells in media with galactose instead of glucose. Galactose is unable to provide sufficient levels of ATP from glycolysis, so cells are forced to depend on OXPHOS (Aguer et al., 2011; Robinson et al., 1992). Thus, ND1 mutant cells in galactose media die within 72 hr while ND1 control cells survive (Figure 1E and Figure S1A, available online). The genome-wide CRISPR screen was performed with ND1 mutant cybrids to discover genes whose loss of function would confer cell viability under galactose conditions, presumably by boosting mitochondrial OXPHOS capacity. The screen employed a validated pooled lentiviral sgRNA library targeting 18,675 genes in the human genome with coverage of four sgRNAs per gene (Doench et al., 2016) (Figure 1E). We scored sgRNAs for enrichment within the pool following growth in galactose media (Figure 1F). Remarkably, in alignment with the chemical screen, one of the highest scoring genes with high fold enrichment and statistical significance was the bromodomain-containing protein 4 (BRD4)—a target that is inhibited by I-BET 525762A. To further confirm these results, we repeated the screen using more stringent conditions, challenging the cells with a second round of growth in galactose media to select persistently galactose-resistant cells. Again, BRD4 emerged as one of the highest scoring genes (Figures S1B and S1C). Together, potent hits from these two independent and unbiased screens (chemical and genome-wide CRISPR library) converging to BRD4 strongly points toward BRD4 inhibition as a mechanism to increase OXPHOS proteins (COX5a) and rescue bioenergetic defects caused by mitochondrial CI mutations.

Bromodomain Inhibition Enhances OXPHOS Protein Activity and Protects against Cell Death in CI-Defective Cells

To validate and extend the impact of bromodomain inhibition on mitochondrial energetic function, we tested its effects on OXPHOS protein levels in ND1 mutant cybrids. I-BET 525762A increased COX5a and an array of other OXPHOS protein levels in a dose-responsive manner (Figures 2A and 2B) with increases persisting at the whole-complex level (Figure 2C and Figure S2A). Additional BRD4 inhibitors with diverse chemical structures, such as JQ1 (Filippakopoulos et al., 2010), GSK1210151A (Dawson et al., 2011), and MS436 (Zhang et al., 2013), also increased COX5a protein levels (Figure 2D). Indeed, comprehensive proteomics analysis indicated that I-BET treatment significantly enhanced proteins involved in mitochondrial OXPHOS, translation, and organization (Figure 2E; Table S1). The increase in protein levels proved to be functional, as treatment with the I-BET 525762A compound enhanced both total oxygen consumption and the activity of ETC complexes II (CII) and IV (CIV) (Figures 3A–3C). I-BET 525762A treatment did not alter glycolysis as measured by ECAR, and mitochondrial complex activity for complex III (CIII) and complex V (CV) was unchanged (Figures S2B–S2D). To assess whether the increase in mitochondrial oxidative capacity from bromodomain inhibitors was sufficient to rescue cell death caused by mitochondrial bioenergetic deficits, ND1 mutant cells were cultured in galactose instead of glucose. Vehicle-treated cells died when exposed to galactose for 72 hr, but strikingly, treatment with I-BET 525762A or other bromodomain inhibitors rescued galactose-induced cell death, and cells proliferated for several passages (Figures 3D, 3E, S1A, and S2E). Interestingly, the bromodomain inhibitor also prevented galactose-induced cell death and enhanced oxygen consumption in other mitochondrial CI-deficient human cybrid cells such as Leber hereditary optic neuropathy (LHON), which carries the G14459A mutation in ND6 (Jun et al., 1994) (Figures 3F and 3G) and knockdown of two different CI subunits, NDUFS3 and NDUFV2 (Figure 3H). The energetic rescue by the bromodomain inhibitor was observed not only in CI mutant cybrid cells but also in fibroblasts derived from patients harboring a mutation in ACAD9, a chaperone protein necessary for CI activity (Nouws

(D) Chemical structure of the I-BET 525762A compound with COX5a fluorescent values below.

(E) Workflow of genome-wide CRISPR screen.

(F) Scatterplot of two replicate CRISPR screens showing enrichment of sgRNAs after galactose treatment. Points highlighted in red represent the different sgRNAs for BRD4. See also Figure S1.

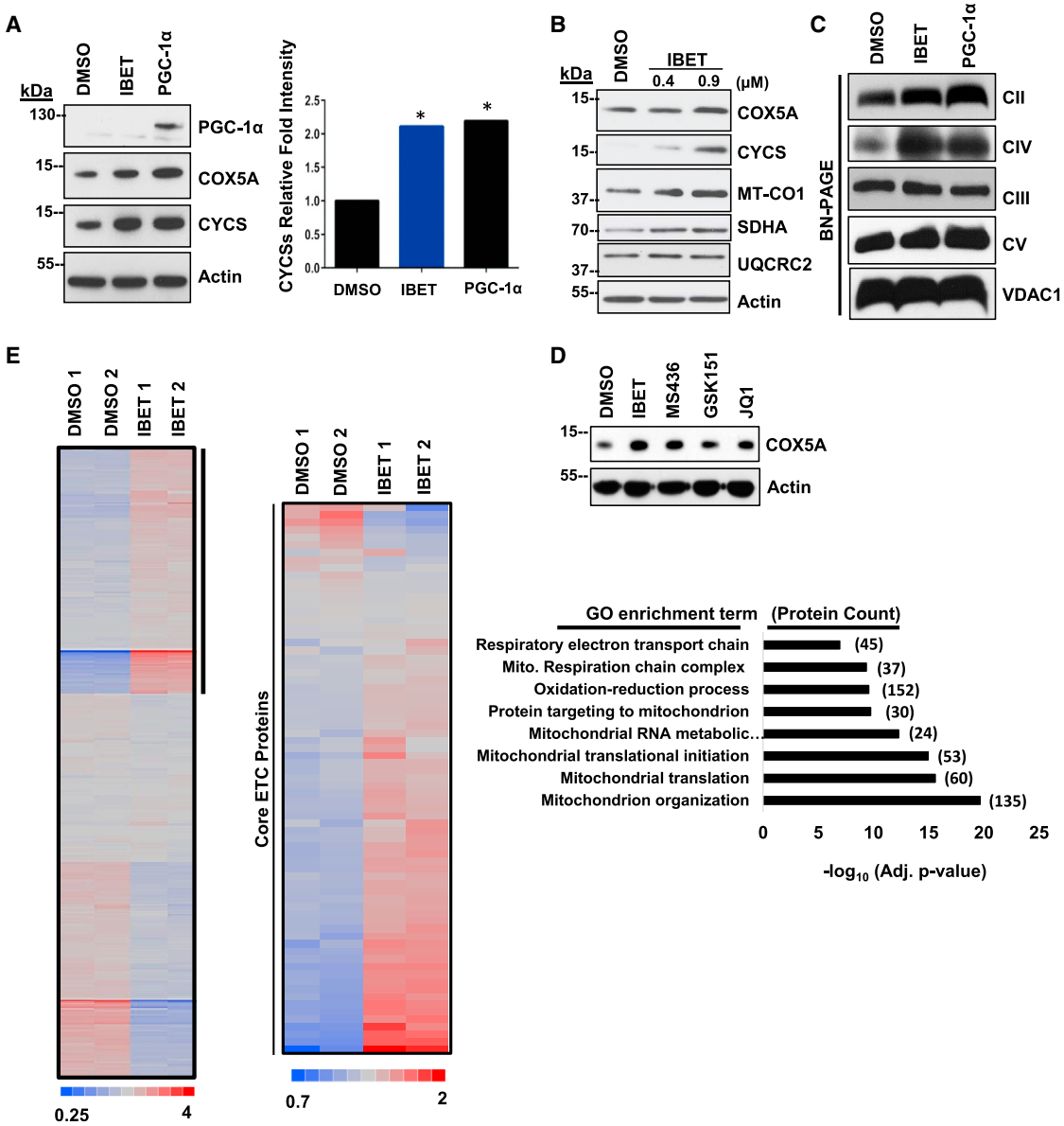

Figure 2. Bromodomain Inhibition Increases OXPHOS Proteins and Remodels the Mitochondrial Proteome

(A) I-BET 525762A increases OXPHOS proteins (left). Cycs densitometry of IBET-treated ND1-mutated cybrids is comparable to PGC-1α-positive control (right).

(B) I-BET 525762A increases OXPHOS proteins in a dose-responsive manner.

(C) Blue native PAGE I-BET 525762A treatment increases OXPHOS complex levels.

(D) Diverse BRD4 inhibitors increase OXPHOS proteins. Immunoblots shown are representative of greater than three independent experiments.

(E) Proteomic heatmap of the hierarchical clustering of all proteins (~4,800) in duplicate (left). I-BET 525762A-upregulated proteins (> 1.4-fold) are indicated (vertical black bar). Proteomic heatmap indicating that I-BET treatment in ND1-mutated cybrids increases core ETC proteins compared to control (middle). Gene Ontology (GO) categories from upregulated proteins enriched after Benjamin-Hochberg correction are indicated to the right. Corresponding $\log_{10}$ p values (x axis) and the number of proteins (in parentheses) are listed. See also Figure S2 and Table S1.

et al., 2010) (Figure 3I). I-BET 525762A also displayed rescue in cells that were chemically inhibited by rotenone (Figure 3J). I-BET-mediated rescue was only observed in CI-deficient cells. Other complex-deficient cells such as MELAS (mitochondrial encephalomyopathy and stroke-like episodes A3243G-Leucine tRNA), MERRF (myoclonic epilepsy and ragged red fibers A8344G-Lysine tRNA), COX10 KO (heme A:farnesyltransferase cytochrome *c* oxidase assembly factor), and Reiske KO (CIII assembly protein) displayed no rescue when cultured in galactose media despite comparable basal oxygen consumption levels (Figures S2F and S2G). These results indicate that bromodomain inhibitors remodel the mitochondrial proteome, increasing oxidative activity and readjusting bioenergetic defects caused by CI deficiency.

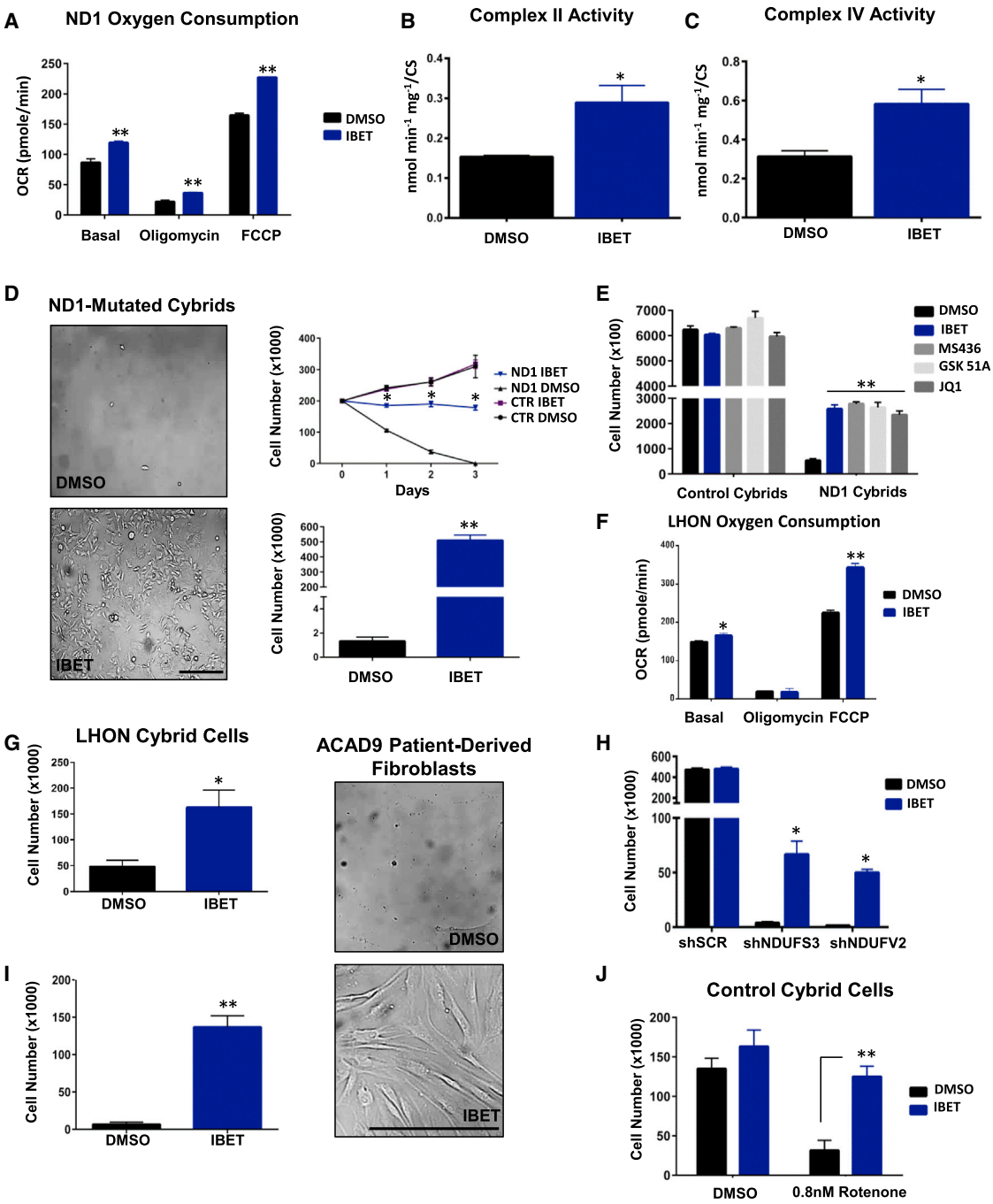

Figure 3. Bromodomain Inhibition Increases Mitochondrial Bioenergetics and Protects against Galactose-Induced Cell Death

(A–C) Total oxygen consumption (A) as well as CII (B) and CIV (C) activities are increased with I-BET 525762A. CS indicates citrate synthase.

(D) I-BET 525762A protects against galactose-induced cell death. Microscopy image (left) is representative of at least three independent experiments, and time course (top right) and cell number quantification (bottom right) are the mean ± SEM, n = 3. Asterisks denote *p < 0.05 or **p < 0.01 via Student's t test. The horizontal black scale bar represents 200 μm.

(E) Different BRD4 inhibitors rescue ND1-mutated cybrids from galactose-induced cell death.

(F and G) I-BET 525762A enhances oxygen consumption in ND6-mutated LHON cybrids (F) and protects against galactose-induced cell death (G).

(H) I-BET 525762A rescues galactose-induced cell death in CI knockdown control cells.

(I) I-BET 525762A protects against galactose-induced cell death in ACAD9 human-patient-derived fibroblasts. Microscopy image (right) is representative of at least three independent experiments, and cell number quantification (left) is the mean ± SEM, n = 3. The horizontal black scale bar represents 200 μm.

(J) I-BET 525762A protects against galactose-induced cell death in control human cybrids with rotenone-induced CI deficiency. Data represent the mean ± SEM, n = 3. Asterisks denote *p < 0.05 or **p < 0.01 via Student's t test. All I-BET and BRD4 inhibitor treatments are delivered at final concentration of 0.9 μM. See also Figure S2.

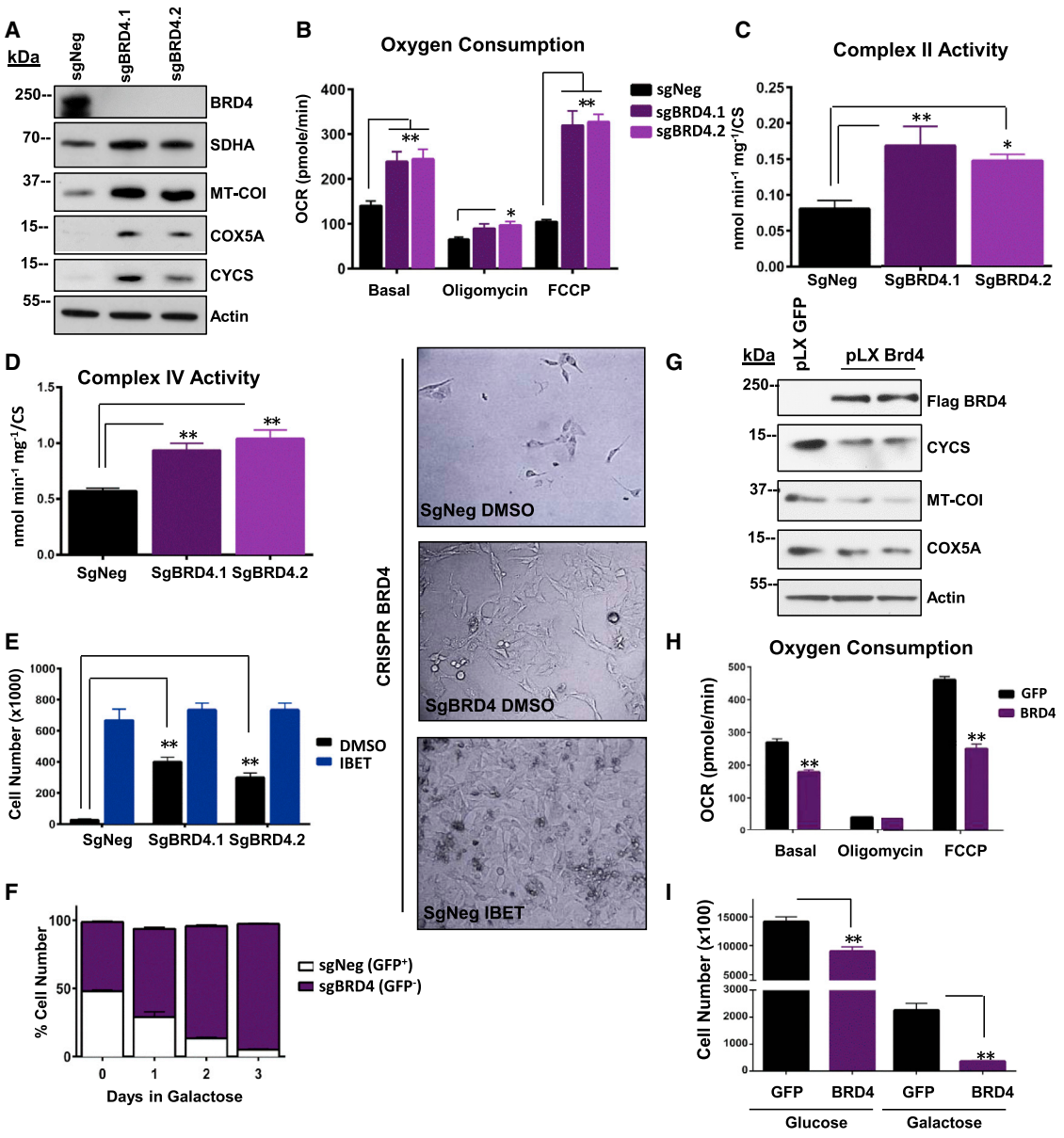

Figure 4. Loss of Function of BRD4 Enhances Mitochondrial Bioenergetics while Gain of Function Impairs OXPHOS Capacity

(A) CRISPR ablation of BRD4 enhances OXPHOS proteins.

(B) Total oxygen consumption rates are increased in sgBRD4 ND1 human cybrids compared to sgNeg control.

(C and D) Complex II (C) and complex IV (D) activities are increased in sgBRD4 ND1 human cybrids compared to sgNeg controls. CS indicates citrate synthase.

(E) Representative microscopy image (right) of sgNeg, sgBRD4, and IBET-treated sgNeg ND1 mutant cybrids cultured in galactose for 72 hr. Cell quantification after 72 hr in galactose media is indicated to the left. The horizontal black scale bar represents 200 μm.

(F) Competition assay. GFP-negative sgBRD4 is more abundant that GFP-positive sgNeg in ND1-mutated cybrids.

(G) BRD4 overexpression in control human cybrids reduces OXPHOS proteins.

(H) Total oxygen consumption is reduced with BRD4 overexpression in control human cybrids.

(I) Control human cybrids are sensitized to galactose-induced cell death with BRD4 overexpression. Immunoblots shown are representative of at least three independent experiments, and all other experiments are the mean ± SEM, n = 3. Asterisks denote *p < 0.05 or **p < 0.01 via Student's t test. All I-BET treatments are delivered at final concentration of 0.9 μM. See also Figure S3.

BRD4 Controls the Mitochondrial OXPHOS Program

We next investigated whether abrogation of BRD4 could increase mitochondrial function in ND1 mutant cells. BRD4 protein was ablated using different sgRNAs and shRNAs; consistent with the I-BET 525762A treatment, sgBRD4-expressing guides and shBRD4 constructs enhanced OXPHOS protein levels (Figure 4A and Figure S3A). In addition, total oxygen consumption and respiratory CII and CIV activities were increased (Figures

4B–4D and Figure S3B). Consistent with I-BET treatment, CIII and CV activities were unaltered in sgBRD4 guides (Figures S3C and S3D). As predicted based on the CRISPR screen, BRD4-ablated cells were protected from galactose-induced cell death (Figure 4E). These results were confirmed using selective competition between sgNeg (GFP+) and sgBRD4 (GFP−)-expressing ND1 mutant cells. After 72 hr with galactose, 98% of the surviving cells were GFP−, indicating that BRD4 ablation conferred a selective advantage under these energy-restricted conditions (Figure 4F). To further support the BRD4 function in mitochondrial energetics, we overexpressed BRD4 in control human cybrid cells. Ectopic expression of BRD4, conversely to its ablation or inhibition, reduced OXPHOS protein levels and decreased total oxygen consumption compared to the levels found in controls (Figures 4G and 4H). In addition, increased expression of BRD4 in control cybrids impaired cell growth under galactose conditions (Figure 4I). These findings indicate that changes in the expression of BRD4 protein alter mitochondrial energetic capacity and cell survival under nutrient conditions that require OXPHOS.

Displacing the BRD4 Protein from Nuclear-Encoded OXPHOS Promoters Increases OXPHOS Genes

We next evaluated the mechanisms whereby BRD4 inhibition increases mitochondrial oxidative function. Bromodomain proteins bind to chromatin through interaction with histone-acetylated lysines (Dhalluin et al., 1999), and I-BET-525762A disrupts this interaction by occupying the ε-amino lysine pocket, leading to direct bromodomain protein displacement from chromatin (Nicodeme et al., 2010). To assess whether BRD4 localizes at promoters of nuclear-encoded mitochondrial genes, we analyzed published datasets containing BRD4 ChIP sequence results (Anders et al., 2014). Genes containing BRD4-binding peaks within 1,000 bp upstream and downstream of the transcription start site (TSS) were identified using a BETA-minus algorithm and were functionally classified using gene ontology and Panther algorithm (Wang et al., 2013; Zhang et al., 2008). Interestingly, these data displayed BRD4 occupancy at promoters of genes linked to the OXPHOS pathway comparable to the BRD4 occupancy found in the control gene *CCND2* (Anders et al., 2014) (Figures S4A and S4B). These findings were validated in ND1 mutant cells through analysis of BRD4 occupancy at promoters of nuclear-encoded mitochondrial genes, including *SDHD*, *CYCS*, and *COX5A*. As expected, BRD4 inhibition by I-BET-525762A decreased BRD4 occupancy at these promoters (Figures 5A and S4C). Intriguingly, displacement of BRD4 by the I-BET compound led to enhanced occupancy of PGC-1α, a transcriptional coactivator that binds to promoters and activates expression of mitochondrial genes (Charos et al., 2012) (Figure 5B). Much like the BRD4 inhibitor, ectopic expression of PGC-1α strongly displaced BRD4 from these promoters (Figure 5C). Consistent with these results, expression of mitochondrial genes was increased in ND1 mutant cells when BRD4 was displaced from OXPHOS gene promoters by I-BET 525762A (Figures 5D and S4D) or depleted with CRISPR or shRNA (Figures 5E and S4E). This is in contrast to the BRD4 target *CCND2*, a gene repressed by the BRD4 inhibitor (Figure S4D). Conversely, ectopic expression of BRD4 decreased the expression of OXPHOS genes (Figure 5F). To further confirm these findings, PGC-1α was depleted from ND1-mutated cybrids. PGC-1α knockdown prevented the I-BET-mediated increase in mitochondrial transcripts and partially blunted the rescue from galactose-induced cell death (Figures 5G and 5H). These data suggest that BRD4 occupancy at nuclear-encoded promoters regulates the expression of mitochondrial genes and prevents PGC-1α, and likely other activators, from binding.

Inhibition of BRD4 Rewires Bioenergetic Metabolism through CII to Overcome CI Deficiency

To gain additional insight into the mechanism of I-BET-mediated enhancement of OXPHOS bioenergetic capacity and protection from galactose-induced cell death, a comprehensive metabolomics analysis was performed (Figure 6A). Intriguingly, metabolites such as dihydroxyacetone phosphate (product of mitochondrial glycerol-3-phosphate dehydrogenase) and fumarate (product of succinate dehydrogenase) were significantly enhanced in I-BET-treated ND1 cybrids compared to DMSO controls (Figures 6A–6C). Also interestingly, these two enzymes are able to bypass the defective CI in the ND1-mutated cells by supplying electrons via $FADH_2$ to CII and CoQ to maintain ATP levels. Indeed, I-BET 525762A treatment in ND1 human cybrids prevented galactose-induced declines in the energetic metabolite ATP and normalized NADH (which accumulates due to CI deficiency (Karamanlidis et al., 2013) to similar levels as control cells (Figures 6D, 6E,S5A, and S5B). Consistent with increases in metabolites converging to CII and CoQ, FAD levels were elevated in cells treated with the bromodomain inhibitor (Figure 6F), suggesting increased utilization of $FADH_2$. Given that galactose-derived pyruvate is not an optimal substrate for oxidation due to the defective CI, we speculated that the increase in oxygen consumption in I-BET-treated ND1 mutant cybrids was accomplished by oxidizing alternative energy sources such as glutamine, which enters the I-BET-enhanced CII via the TCA cycle. Indeed, I-BET 525762A treatment increased ^{14}C glutamine oxidation and was able to moderately prevent cell death when glutamine was the main carbon source (Figures 6G and 6H). In addition, I-BET treatment enhanced genes in the glutamine utilization pathway (Figure S5C). In contrast, I-BET 525762A failed to prevent galactose-induced cell death when glutamine was either removed or the glutaminase enzyme (which catalyzes glutamine to glutamate conversion) was chemically blocked (Figures 6H and 6I). Taken together, these results show that I-BET 525762A augments both glutamine oxidation and the metabolites that generate $FADH_2$. $FADH_2$ can be oxidized through increased CII and CIV activities to produce ATP and rescue cell death. To assess whether CII is required for I-BET 525762A-induced survival, we genetically abrogated CII function using two shRNAs targeting the SDHA subunit to disrupt both the ETC and TCA cycle. Interestingly, I-BET 525762A-mediated cell rescue was considerably minimized in knockdown cells placed under galactose for 72 hr (Figure 6J). To further test whether increases in mitochondrial oxidative activity—mediated via BRD4 inhibition—are necessary to maintain bioenergetics and cell survival in CI-deficient cells, we used two different approaches to completely disrupt mitochondrial respiration: chloramphenicol, an antibiotic that blocks mitochondrial protein

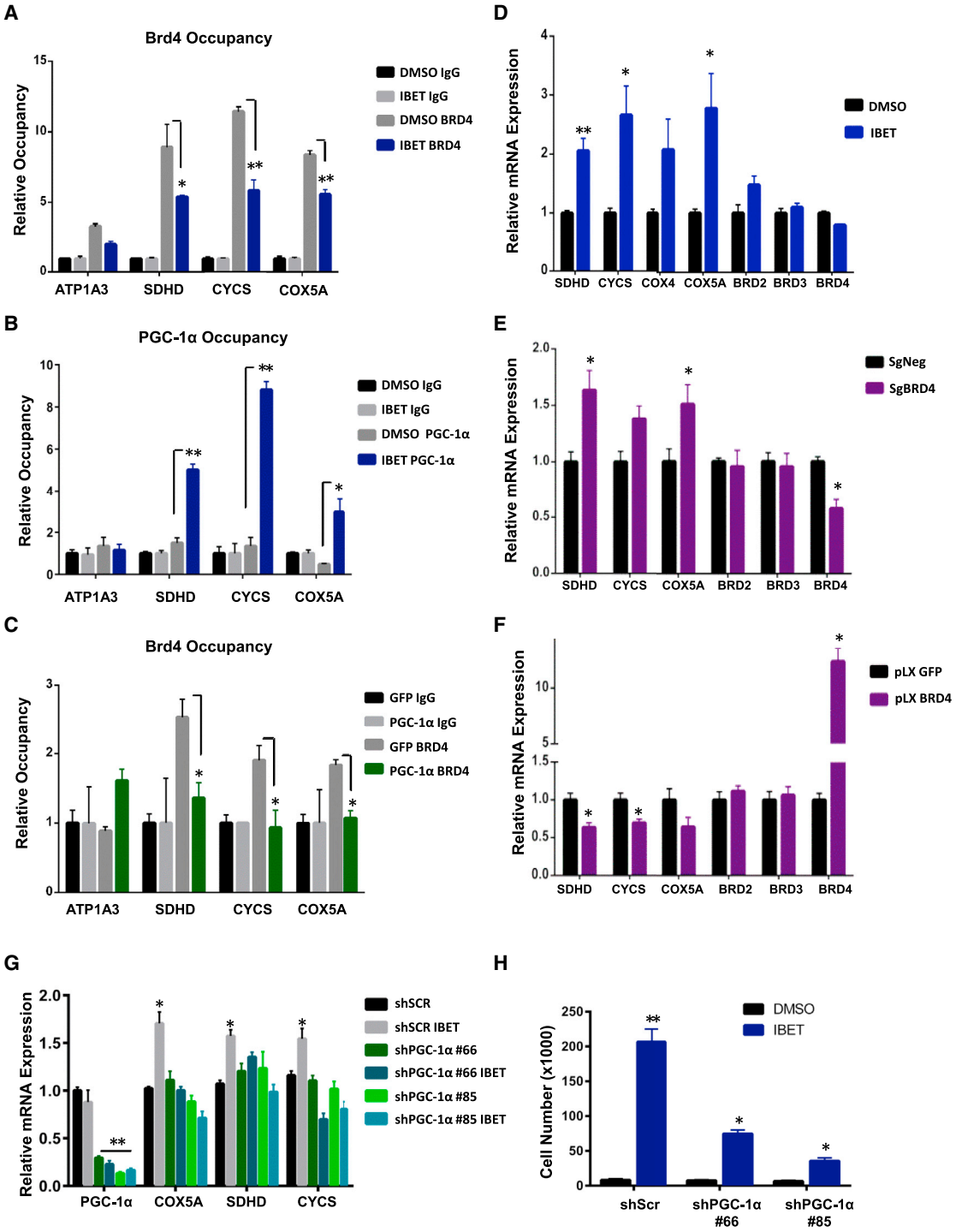

Figure 5. IBET 525762A Treatment Displaces BRD4 from OXPHOS Promoters and Increases Transcription in CI-Deficient Cells

(A) ChIP assay. I-BET 525762A displaces BRD4 from nuclear-encoded mitochondrial promoters. Negative control promoter region Atp1A3 is unaltered.

(B) ChIP assay. PGC-1α occupancy increases upon I-BET 525762A-mediated BRD4 displacement.

(C) ChIP assay. BRD4 is displaced upon adPGC-1α overexpression. Data are representative of mean ± SEM, n = 3. Asterisks denote *p < 0.05 or **p < 0.01 via Student's t test.

(D–F) I-BET 525762A-mediated displacement of BRD4 (D) and ablation of BRD4 by CRISPR (E) increases expression of OXPHOS genes, while overexpression of BRD4 decreases OXPHOS gene expression (F). Data represent mean ± SEM, n = 3. Asterisks denote *p < 0.05 or **p < 0.01 via Student's t test.

(G and H) ShPGC-1α KD in ND1-mutated cybrids fails to increase OXPHOS transcripts (G) and partially blunts rescue from galactose-induced cell death (H) when treated with I-BET 525762A. See also Figure S4.

translation, and ethidium bromide (EtBr), which causes mitochondrial DNA depletion. In both cases, the levels of MT-CO1, a mtDNA encoded-protein translated by mitochondrial ribosomes and essential for respiration (Tsukihara et al., 1996), were undetectable (Figures S5D and S5E). I-BET 525762A failed to rescue galactose-induced cell death in chloramphenicol or EtBr-treated cells (Figures S5D and S5E). In summary, these results indicate that I-BET 525762A-induced mitochondrial respiration is necessary to rescue the bioenergetic defects and maintain survival in human cybrids with mitochondrial CI mutations.

DISCUSSION

Cells harboring mitochondrial mutations in CI fail to cope with excessive energetic demands and undergo cell death. In principle, a metabolic reprogramming that increases ETC activity and bypasses mutated CI could rescue the bioenergetic defects and maintain cell survival. Our studies have identified that the inhibition of BRD4 plays a regulatory role in this metabolic reprogramming. BRD4 binds to nuclear-encoded mitochondrial genes, and its inhibition efficiently rewires cellular energetics caused by CI defects (Figure 6K). BRD4 inhibition remodels the mitochondrial proteome and causes changes in metabolic routes that increase and utilize $FADH_2$, transferring electrons to the respiratory chain downstream of CI and increasing ATP.

Metabolic strategies to reprogram and bypass ETC defects in mitochondrial disease mutations have been based on CoQ derivatives or NAD^+ precursors, but their efficacy has been limited, particularly in CI deficiencies (Pfeffer et al., 2013). Bromodomain inhibitors might overcome these metabolic limitations by acting through a broad, mitochondrial-based gene-expression program that generates energetic, efficient mitochondria to bypass CI defects. Thus, coordinated increases in CII and CIV through BRD4 inactivation would allow oxidation of substrates such as glutamine, fatty acids, or branched-chain amino acids, increasing the $FADH_2$ that will enter the ETC at CII or CoQ sites. Mounting evidence demonstrates that oxidizing glutamine can serve as the major substrate for energy production (Lu et al., 2010; Reitzer et al., 1979). We show that, in addition to OXPHOS genes, transcripts linked to glutamine metabolism are also upregulated by I-BET treatment, which supports the role of glutamine catabolism and ATP production by BRD4 inhibition in CI-deficient mutant cells. Consistent with this demonstration of energetic and metabolic rescue via BRD4 inhibition, previous studies have corrected CI deficiency by introducing the *Saccharomyces cerevisiae* NADH-quinone oxidoreductase (NDI1) protein to bypass the defective CI and increase OXPHOS and ATP production from CIII and CIV (Bai et al., 2001).

A metabolic hallmark of CI malfunction is NADH accumulation caused by reduction in CI-dependent NADH reductase activity (Karamanlidis et al., 2013). Increased NADH might alter catalytic activity of NAD^+-dependent enzymes such as sirtuins, TCA cycles, or glycolytic dehydrogenases (Nunnari and Suomalainen, 2012). We show that NADH levels are partially restored after bromodomain inhibition and may contribute to bioenergetic rescue in CI-deficient cells. Although a complete mechanism of how bromodomain inhibition normalizes NADH is unknown, some putative candidates, such as mitochondrial GPD2 (glycerol phosphate dehydrogenase), emerged in the proteomic analysis and were elevated in isolated mitochondria from I-BET-treated samples (Figure 2E). GPD2 is a mitochondrial, membrane-bound protein that forms part of the glycerol-phosphate shuttle that transfers cytosolic NADH to $FADH_2$ and bypasses CI inhibition (Mráček et al., 2013). The accumulated mitochondrial NADH caused by the defective CI could be transported to the cytosolic compartment using the malate-aspartate pathway to provide the substrate for the glycerol-phosphate shuttle (Gut and Verdin, 2013). Furthermore, metabolomic analysis shows that several metabolites related to cellular redox states and antioxidant response were increased by I-BET treatment. Deleterious phenotypes caused by CI failure have been shown to arise when increased reactive oxygen species (ROS) levels and compromised ATP generation are present (Haefeli et al., 2011). Thus, increases in ROS detoxification through bromodomain inhibition might also contribute to the bioenergetic rescue and survival caused by defective CI.

In these studies, I-BET 525762A was extremely effective in increasing OXPHOS capacity and promoting survival under nutrient-restrictive galactose conditions in cells with CI disruption. I-BET, however, was unable to rescue MELAS and MERRF cybrid cells harboring other mitochondrial complex mutations. It would appear that I-BET 525762A can only provide rescue when the defective complexes occur at an early point in the ETC, such that these complexes can be bypassed to boost residual OXPHOS capacity. Given that CIV levels and activity are also increased upon I-BET treatment, it is conceivable that BRD4 inhibition, similar to PGC-1α expression (Srivastava et al., 2009), would positively impact mutations in which CIV is not fully ablated. A basal threshold of ETC activity seems to be indispensable for bromodomain inhibitors to rescue bioenergetic defects and promote survival, as shown in the experiments using chloramphenicol or EtBr cells (Figures S5D and S5E).

BRD4 is a chromatin-bound transcriptional regulator linked to expression of genes associated with different biological processes, including tumor progression or inflammation (Baratta et al., 2015; Huang et al., 2009). However, how and whether these programs integrate with mitochondrial energetics and metabolic control is unknown. As a scaffolding protein, BRD4 recruits activator or repressor complexes to target promoters. Its activator function has been extensively characterized through interactions with pTEFb, Mediator, and JMJD6, all of which promote RNA polymerase II (Pol II) elongation (Liu et al., 2013). When displaced from chromatin by bromodomain inhibitors, this corresponds to a decrease of target genes such as MYC (Delmore et al., 2011). BRD4, however, can also be incorporated into repressor complexes as demonstrated in the human papillomavirus (HPV) system (Wu et al., 2006). Chromatin-bound BRD4 recruits and binds to the HPV viral protein E2 and prevents the binding of TFIID and Pol II to the target promoters leading to gene repression. Genome-wide RNA sequencing analysis of BRD4 abrogation reveals a dual role of BRD4, either in increasing or decreasing mRNA transcripts (Shu et al., 2016). BRD4 occupies nuclear-encoded mitochondrial promoters, and its inhibitor, I-BET 525762A, significantly reduces BRD4 occupancy at the mitochondrial promoters. Our results indicate that I-BET-mediated gene repression occurs almost immediately after I-BET treatment; however, activation of OXPHOS genes occurs

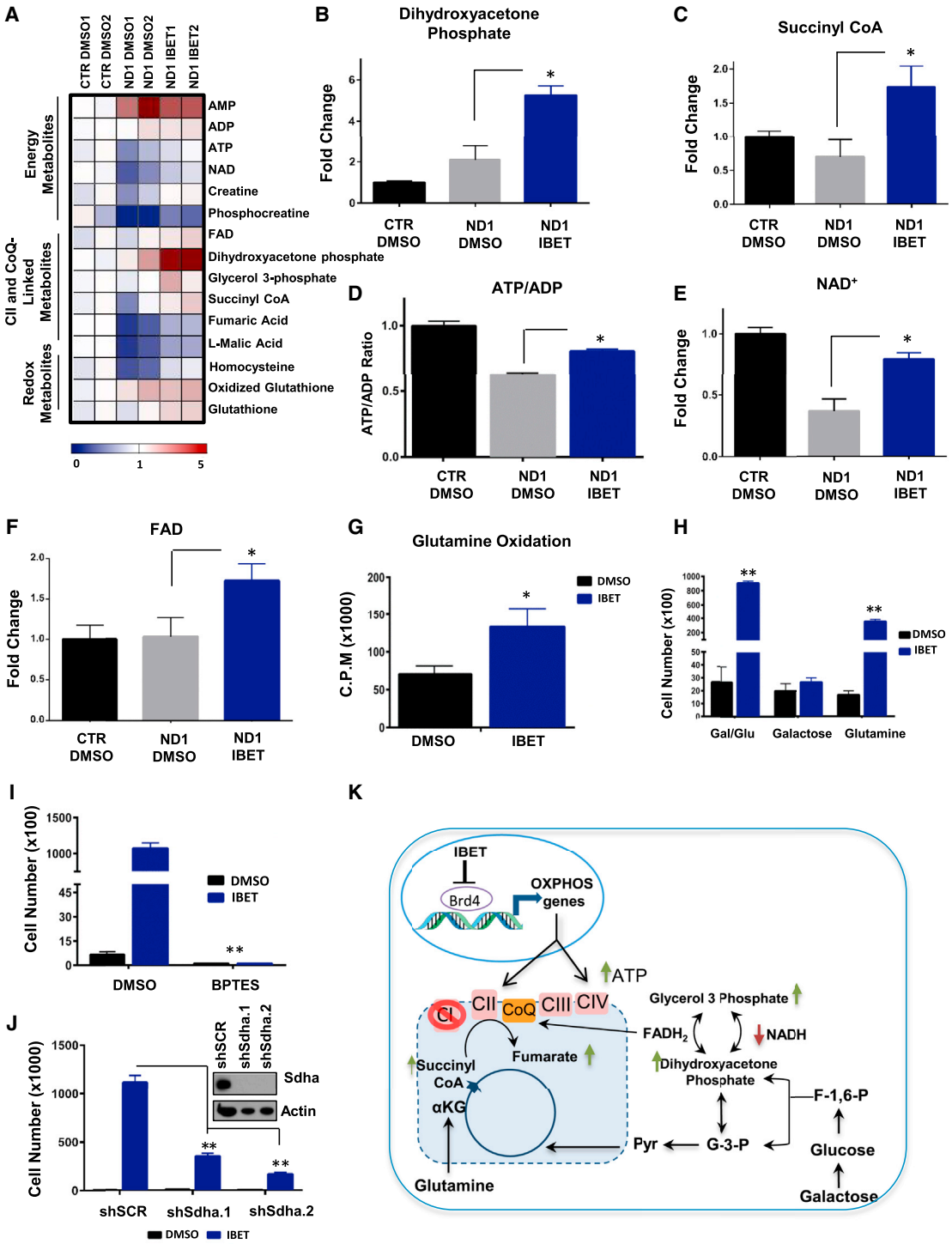

Figure 6. I-BET 525762A Treatment Rewires and Enhances Metabolic and Energetic States of CI-Deficient Cells

(A) Metabolomics heatmap in control and ND1-mutated cybrids in duplicate. I-BET 525762A-treated ND1-mutated human cybrids increase metabolites linked to energy, CII, CoQ, and redox metabolism.

(B and C) I-BET 525762A treatment increases dihydroxyacetone phosphate (B) and succinyl CoA (C) metabolite levels.

(D–F) I-BET 525762A treatment increases ATP (D), NAD^+ (E), and FAD (F) metabolite levels in ND1-mutated cybrids. Data represent mean ± SEM, n = 2.

(G) ^{14}C Glutamine oxidation is enhanced by I-BET 525762A treatment in ND1-mutated cybrids.

(H) I-BET-mediated galactose rescue is blunted when glutamine is removed from culture media in ND1-mutated cybrids.

(legend continued on next page)

over a longer time point. These time-dependent differences between I-BET-mediated gene repression and activation could be attributed to the difference in the dynamic recruitment and composition of transcription factors to these promoters. Increased transcription of OXPHOS genes suggests that BRD4 competes with a potent activator and may also be associated with a repressor protein. Given that (1) the displacement of BRD4 from nuclear-encoded mitochondrial promoters increased the occupancy of potent transcriptional activators such as PGC-1α (Figure 5B), and (2) I-BET-mediated increase in OXHPOS genes and cell survival under galactose conditions was greatly impeded with PGC-1α knockdown, our current data would seem to suggest a competition model (Jang et al., 2005; Wallberg et al., 2003). The involvement of a repressor protein or an alternate mechanism, however, may also be plausible, as there was partial, albeit significantly blunted, rescue in ND1-mutated cybrids upon I-BET treatment in the context of PGC-1α knockdown under galactose conditions.

The ability of bromodomain inhibition to correct bioenergetic defects caused by deficient CI activity through different metabolic strategies may be employed as a possible avenue to treat mitochondrial and neurodegenerative diseases in which the inhibition of CI has been associated with disease progression.

EXPERIMENTAL PROCEDURES

High-Throughput Chemical Screening

A total of 1.0×10^6 human ND1 mutant (3796 A > G) cybrid cells were seeded in a 384-well plate (Corning, 3712) and incubated for 24 hr at 37°C with 5% CO_2. Positive control wells (column 24) were infected with adenoviral Pgc-1α and negative control lanes (column 23) and received 0.3% DMSO. Following a media change, chemical compounds were added to experimental wells via pin transfer, and cells were incubated with compound for 24 hr at 37°C with 5% CO_2. The In-Cell ELISA (ICE) protocol was then performed. Cells were washed with PBS (HyClone) prior to being fixed with 4% paraformaldehyde (Santa Cruz, sc281692) and permeabilized with 0.2% Triton X-100 (Sigma, X100). Cells were washed twice with PBS and treated with Alexa Fluor 680 succinimidyl ester (Thermo Fisher, A37567) for internal control before blocking with blocking buffer (LI-COR, 927-40000) and incubating with COX5a antibody (Abcam, ab110262) for 16 hr at 4°C. Cells were then washed with 0.2% Triton X-100 in PBS before the addition of the secondary anti-mouse IRDye 800CW antibody (LI-COR, 926-32212) for 1 hr. Fluorescence was then quantified using the LI-COR Aerius instrument and software. Values in the 800 channel were normalized to the respective internal control (700 channel). Normalized data were then expressed as a ratio to the average positive control (set to 100%). Further details for screening methods are available on request.

Design and Creation of the CRISPR Library

The human genome-wide lentipool CRISPR library was designed and created as previously described (Doench et al., 2016)

Genome-wide CRISPR Screen

A total of 1.1×10^8 human ND1 mutant (3796 A > G) cybrid cells, which stably expressed Cas9, were seeded in 38 150 mm × 25 mm dishes (3.0×10^6 per dish). Cells were infected with the lentipooled library to achieve a 30%–50% infection efficiency, corresponding to a multiplicity of infection (MOI) of ~0.3–0.5. Media was changed 24 hr later, and 0.5 ug/mL puromycin was added 48 hr later and selected for 7 additional days. Cells were trypsinized and separated into 150 mm × 25 mm dishes (4.0×10^6 per dish). The next day, cells were washed twice with PBS, and media was changed to non-glucose DMEM with glutamine (4 mM) supplemented with 10 mM galactose (Sigma D7050), 10% FBS, 1% P/S, and 1 mM pyruvate. Cells were cultured for 5 days in galactose media. After 5 days, galactose media was replaced with high-glucose DMEM in order to expand the remaining cells. Cells were maintained and passaged in glucose DMEM for 2 weeks before genomic DNA was isolated from two biological replicates using QIAamp DNA mini kit (QIAGEN 51304). A third independent experiment was performed as described above, but this time cells were challenged with two rounds of 10 mM galactose treatment.

Illumina Sequencing

Samples were submitted and sequenced at the Broad Institute using a HiSeq2000 (Illumina) as previously described (Doench et al., 2016).

Screen Analysis and Statistics

For analysis, the $\log_2$-fold change of each sgRNA was normalized to the starting plasmid DNA (pDNA) pool for each biological replicate unless otherwise stated. Each sgRNA was ranked by $\log_2$-fold change, and this number was then divided by the total number of sgRNAs in the pool to determine a percent-rank value. These percent-rank values from two biological replicates were represented as scatterblots. A third independent experiment using more stringent culture conditions was analyzed equally but represented as a bar graph. In order to perform RIGER and STARS analysis, the percent-rank values across subpools were merged. STARS analysis allows the calculation of p values, FDR, and q value (corrected FDR) for hit genes. STARS is written in Python, and is publicly available: http://www.broadinstitute.org/rnai/public/software/index.

Cell Culture and Treatments

All cell lines were maintained in high-glucose DMEM (HyClone), 10% FBS, and 1% P/S at 37°C and 5% CO_2. For compound treatment, cells were seeded at a density of 1.0×10^5 cells per well in a 6-well plate (Falcon, 353046) and allowed to adhere for 24 hr before treatment with 0.9 μM I-BET 525762A, JQ1, GSK1210151A, MS436 chemical compounds, or vehicle control for 24 hr in high-glucose DMEM. For galactose experiments, cells were seeded in 6-well plates grown in high-glucose DMEM, 10% FBS, and 1% P/S at 37°C and 5% CO_2 for 24 hr to allow cells to adhere. Cells were then washed twice with PBS and media was changed to DMEM with no glucose but supplemented with 4 mM glutamine (HyClone) and 10 mM galactose (Sigma G0750), 10% FBS, and 1% P/S. Cells were incubated in galactose-containing media ± 0.9 μM I-BET 525762A for 72 hr with daily media changes. Galactose media included 50 μM BPTES (Sigma SML0601) for experiments assessing glutamine requirement. Cells were then trypsinized and quantified using a hemocytometer (NanoEnTek, DHC-N01). Generation of RhoO cells involved treating control cybrids with high-glucose DMEM supplemented with 50 ng/mL of ethidium bromide (Bio-Rad 1610433) and 50 μg/mL of uridine (Sigma U3750) for 2 weeks with media changes every 48 hr. To abrogate mitochondrial protein translation, control cybrids were treated with high-glucose DMEM supplemented with 40 μM chloramphenicol (Sigma C0378) and 50 μg/mL uridine for 5 days with daily media changes. Mitochondrial protein depletion for both ethidium bromide and chloramphenicol treatments were

(I) Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) at 25 μM, which selectively inhibits Glutaminase (GLS1) to block glutamine to glutamate conversion, abolished the positive effects of I-BET 525762A in ND1-mutated cybrids.
(J) I-BET 525762A displays minimal rescue upon SDHA knockdown. Immunoblot indicates complete SDHA knockdown at the protein level. Data represent the mean ± SEM, n = 3. Asterisks denote *$p < 0.05$ or **$p < 0.01$ via Student's t test. All I-BET treatments are delivered at a final concentration of 0.9 μM.
(K) Model detailing how BRD4 inhibition rescues bioenergetic defects in CI-deficient cells. Arrows indicate metabolites that were increased (green) or decreased (red) upon I-BET 575762A treatment. Abbreviations are as follows: αKG, alpha-ketoglutarate; G-3-P, glyceraldehyde-3-phosphate; F-1-6-P, fructose 1,6 bisphosphate; and CoQ, coenzyme Q. See also Figure S5.

assessed by measuring MT-CO1 protein level. Cells were then placed in galactose media (described above) in the presence or absence of 0.9 μM I-BET for 72 hr. For rotenone treatments, control cybrid cells were seeded in high-glucose DMEM with 0.8 nM rotenone (Sigma 8875) in the presence or absence of 0.9 μM I-BET 525762A for 5 days with daily media changes. Cell quantity was assessed by cell counting after treatment. For selective competition assay, 5 × 10^4 of sgNeg (GFP+) and sgBRD4 (GFP−) ND1 cybrid cells (1 to 1) were plated together in a 6-well plate and cultured in galactose media (previously described). Cells were trypsinized and analyzed by FACS (BD FACS Canto II) daily for 3 consecutive days. The ratio between sgNeg (GFP+) and sgBRD4 (GFP−) was calculated. Transfections for gain- and loss-of-function studies were performed according to the manufacturer's instructions using the polyfect reagent (QIAGEN, 301107). The following constructs were used: pLJM1-sgBRD4.1, 5′-GAGCAGGTATTGCAGTTGGT-3′; pLJM1-eGFP (addgene 19319); pLKO.1 shBRD4, 5′-CCGGCCTGGAGATGACATAGTCTTACTCGAGTAAGACTATGTCATCTCCAGGTTTTTG-3′ (Sigma); pLKO.1 shSDHA.1, 5′-GATTTGCTGATGGAAGCATAA-3′, pLKO.1 shShSDHA.2, 5′-TCGCTATTGCACACCTTATAT-3′; pLKO.1 shNDUFS3, 5′-TTTGTTCCTGCTCGCAATAAC-3′; pLKO.1 shNDUFV2, 5′-CCAGTTGGAAAGTATCACATT-3′, pLKO.1 shPGC-1α 66, 5′-CCGGTATGACAGCTACGAGGAATATCTCGAGATATTCCTCGTAGCTGTCATATTTTTG-3′; pLKO.1 shPGC-1α 85, 5′- CCGGCCGTTATACCTGTGATGCTTTCTCGAGAAAGCATCACAGGTATAACGGTTTTT-3′; pLKO.1 shCTR; pLX-GFP; pLX-BRD4 (Human ORFeome 71377); and lenti-CRISPR v2 (addgene 52951). Guide sequences for Brd4 were as follows: sgBrd4.1, 5′-GAGCAGGTATTGCAGTTGGT-3′; and sgBrd4.2, 5′- ACTGCAATACCTGCTCAGAG-3′.

SUPPLEMENTAL INFORMATION

Supplemental information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.08.023.

AUTHOR CONTRIBUTIONS

P.P., E.B., and J.J.B. conceived the project. P.P. and J.J.B. designed, optimized, and initiated the high-throughput chemical screen. J.J.B., in collaboration with the ICCB, performed the high-throughput chemical screen; processed data; identified and characterized the I-BET 525762A compound; performed oxygen consumption and ECAR analyses; performed galactose rescue experiments of MELAS, MERRF, COX10, Reiske, LHON cybrids, and ACAD9 patient-derived fibroblasts; and performed BRD4 gain-of-function experiments and metabolomics with assistance from the BIDMC Metabolomics Core. P.P. and E.B., with advice and expertise from J.D. and D.E.R., conceived and designed the galactose-based, genome-wide CRISPR screen. E.B., with assistance from L.R.H., performed the genome-wide CRISPR screen. J.D. and D.E.R performed library generation, sequencing, and bioinformatics analysis of the CRISPR sequencing data. E.B. performed mitochondrial activity assays; BN-PAGE; glutamine experiments; loss-of-function experiments of CI and CII; galactose rescue of ND1, CI, and CII cybrid cell lines; and generation and characterization of the shPGC-1α cell lines. R.V. and J.S. were involved in the initial experimental design of the chemical screen and provided ND1 cybrids. E.B. and J.J.B. generated and performed experiments characterizing CRISPR BRD4. J.J.B., F.V., and C.D.J.T. performed the ChIP experiments. M.J., J.A.P., and S.P.G. performed and analyzed the proteomic experiments. J.J.B., E.B., and P.P. wrote the manuscript. M.S.S. critically reviewed data and manuscript.

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